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PTO/SB/05 (11-00)

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# UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. 1.53(b))

Attorney Docket No. 3367

First Inventor Hunt

Title METHODS AND COMPUTER SOFTWARE PRODUCTS FOR  
PREDICTING NUCLEIC ACID HYBRIDIZATION AFFINITY

Express Mail Label No. EL675506548US

## APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☒ Fee Transmittal Form (e.g., PTO/SB/17)  
(Submit an original and a duplicate for fee processing)
2. ☐ Applicant claims small entity status.  
See 37 CFR 1.27.
3. ☒ Specification [Total Pages 47]  
(preferred arrangement set forth below)
  - Descriptive title of the Invention
  - Cross References to Related Applications
  - Statement Regarding Fed sponsored R & D
  - Reference to sequence listing, a table, or a computer program listing appendix
  - Background of the Invention
  - Brief Summary of the Invention
  - Brief Description of the Drawings (if filed)
  - Detailed Description
  - Claim(s)
  - Abstract of the Disclosure
4. ☒ Drawing(s) (35 U.S.C. 113) [Total Sheets 15]
5. Oath or Declaration [Total Pages 3]
  - a. ☒ Newly executed (original or copy)
  - b. ☐ Copy from a prior application (37 CFR 1.63 (d))  
(for a continuation/divisional with Box 18 completed)
  - i. ☐ **DELETION OF INVENTOR(S)**  
Signed statement attached deleting inventor(s)  
named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
6. ☐ Application Data Sheet. See 37 CFR 1.76

## ADDRESS TO:

Assistant Commissioner for Patents  
Box Patent Application  
Washington, DC 20231

7. ☐ CD-ROM or CD-R in duplicate, large table or  
Computer Program (Appendix)
8. Nucleotide and/or Amino Acid Sequence Submission  
(if applicable, all necessary)
  - a. ☐ Computer Readable Form (CRF)
  - b. Specification Sequence Listing on:
    - i. ☐ CD-ROM or CD-R (2 copies); or
    - ii. ☐ paper
  - c. ☐ Statements verifying identity of above copies

## ACCOMPANYING APPLICATIONS PARTS

9. ☒ Assignment Papers (cover sheet & document(s))
10. ☐ 37 C.F.R. §3.73(b) Statement ☐ Power of  
(when there is an assignee) Attorney
11. ☐ English Translation Document (if applicable)
12. ☒ Information Disclosure ☒ Copies of IDS  
Statement (IDS)/PTO-1449 Citations
13. ☐ Preliminary Amendment
14. ☒ Return Receipt Postcard (MPEP 503)  
(Should be specifically itemized)
15. ☐ Certified Copy of Priority Document(s)  
(if foreign priority is claimed)
16. ☐ Request and Certification under 35 U.S.C. 122  
(b)(2)(B)(i). Applicant must attach form PTO/SB/35  
or its equivalent.
17. ☐ Other:

18. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment, or in an Application Data Sheet under 37 CFR 1.76:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP)

of prior application No. \_\_\_\_ /

Prior application information: Examiner \_\_\_\_

Group / Art Unit

For CONTINUATION or DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 5b, is considered a part of the disclosure of the accompanying or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

## 17. CORRESPONDENCE ADDRESS

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Wei Zhou

Registration No. (Attorney/Agent)

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Signature

Date

Nov. 21, 2000

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**FEE TRANSMITTAL  
for FY 2001**

Patent fees are subject to annual revision.

**Complete if Known**

Application Number	
Filing Date	Nov 21, 2000
First Named Inventor	Hunt
Examiner Name	
Group / Art Unit	

**TOTAL AMOUNT OF PAYMENT** (\$) 1532

Attorney Docket No. 3367

**METHOD OF PAYMENT (check one)**1. ☒ The Commissioner is hereby authorized to charge indicated fees and credit any over payments to:Deposit  
Account  
Number

01-0431

Deposit  
Account  
Name

Affymetrix, Inc

☒ Charge Any Additional Fee Required  
Under 37 CFR 1.16 and 1.17  
☐ Applicant claims small entity status.  
See 37 CFR 1.27
2. ☐ Payment Enclosed:
☐ Check ☐ Credit card ☐ Money Order ☒ Other
**FEE CALCULATION (continued)****3. ADDITIONAL FEES**

Fee Code	Large Entity Fee (\$)	Small Entity Fee Code	Small Entity Fee (\$)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	390	216	195	Extension for reply within second month	
117	890	217	445	Extension for reply within third month	
118	1,390	218	695	Extension for reply within fourth month	
128	1,890	228	945	Extension for reply within fifth month	
119	310	219	155	Notice of Appeal	
120	310	220	155	Filing a brief in support of an appeal	
121	270	221	135	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,240	241	620	Petition to revive - unintentional	
142	1,240	242	620	Utility issue fee (or reissue)	
143	440	243	220	Design issue fee	
144	600	244	300	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	130	123	130	Petitions related to provisional applications	
126	180	126	180	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	40
146	710	246	355	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	710	249	355	For each additional invention to be examined (37 CFR § 1.129(b))	
179	710	279	355	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	

Other fee (specify)

\*Reduced by Basic Filing Fee Paid

SUBTOTAL (3)

(\$ 40)

**FEE CALCULATION****1. BASIC FILING FEE**

Large Fee Code	Large Entity Fee (\$)	Small Fee Code	Small Entity Fee (\$)	Fee Description	Fee Paid
101	710	201	355	Utility filing fee	710
106	320	206	160	Design filing fee	
107	490	207	245	Plant filing fee	
108	710	208	355	Reissue filing fee	
114	150	214	75	Provisional filing fee	

SUBTOTAL (1)

(\$ 710)

**2. EXTRA CLAIM FEES**

Total Claims	59	-20**	=	39	X	Fee from below	18	=	Fee Paid	702
Independent Claims	4	-3**	=	1	X	80	=	80		
Multiple Dependent					X		=	0		


Large Fee Code	Large Entity Fee (\$)	Small Fee Code	Small Entity Fee (\$)	Fee Description
103	18	203	9	Claims in excess of 20
102	80	202	40	Independent claims in excess of 3
104	270	204	135	Multiple dependent claim, if not paid
109	80	209	40	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2)

(\$ 782)

\*\*or number previously paid, if greater, For Reissues, see above

**SUBMITTED BY****Complete (if applicable)**

Name (Print/Type)	Wei Zhou	Registration No. Attorney/Agent	44,419	Telephone	408-731-5699
Signature		Date	Nov 21, 2000		

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PATENT

Attorney Docket No. 3367

**PATENT APPLICATION**

**METHODS AND COMPUTER SOFTWARE PRODUCTS  
FOR PREDICTING NUCLEIC ACID HYBRIDIZATION  
AFFINITY**

Inventor:

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5

**METHODS AND COMPUTER SOFTWARE  
PRODUCTS FOR PREDICTING NUCLEIC ACID  
HYBRIDIZATION AFFINITY**

**U.S. GOVERNMENT INTERESTS**

This invention was made with government support under Grant number  
\_\_\_\_\_ from the National Institutes of Health. Accordingly, the U.S. Government  
10 retains certain rights in the invention.

**BACKGROUND OF THE INVENTION**

The present invention relates to methods for analyzing DNA microarray data and  
for designing DNA microarrays.

U.S. Patent No. 5,424,186 describes a pioneering technique for, among other  
15 things, forming and using high density arrays of molecules such as oligonucleotide, RNA,  
peptides, polysaccharides, and other materials. This patent is hereby incorporated by  
reference for all purposes.

Arrays of oligonucleotides or peptides, for example, are formed on the surface by  
sequentially removing a photoremovable group from a surface, coupling a monomer to  
20 the exposed region of the surface, and repeating the process. These techniques have been  
used to form extremely dense arrays of oligonucleotides, peptides, and other materials.  
The synthesis technology associated with this invention has come to be known as  
“VLSIPS™” or “Very Large Scale Immobilized Polymer Synthesis” technology.

Additional techniques for forming and using such arrays are described in U.S.  
25 Patent Nos. 5,384,261, and 6,040,193 which are also incorporated by reference for all

purposes. Such techniques include systems for mechanically protecting portions of a substrate (or chip), and selectively deprotecting/coupling materials to the substrate. Still further techniques for array synthesis are provided in U.S. Application No. 08/327,512, also incorporated herein by reference for all purposes.

5 Nucleic acid probe arrays have found wide applications in gene expression monitoring, genotyping and mutation detection. For example, massive parallel gene expression monitoring methods using nucleic acid array technology have been developed to monitor the expression of a large number of genes (e.g., U.S. Patent Numbers 5,871,928, 5,800,992 and 6,040,138; de Saizieu *et al.*, 1998, Bacteria Transcript Imaging  
10 by Hybridization of total RNA to Oligonucleotide Arrays, NATURE BIOTECHNOLOGY, 16:45-48; Wodicka *et al.*, 1997, Genome-wide Expression Monitoring in *Saccharomyces cerevisiae*, NATURE BIOTECHNOLOGY 15:1359-1367; Lockhart *et al.*, 1996, Expression Monitoring by Hybridization to High Density Oligonucleotide Arrays. NATURE BIOTECHNOLOGY 14:1675-1680; Lander, 1999, Array of Hope, NATURE-GENETICS,  
15 21(suppl.), at 3, all incorporated herein by reference for all purposes). Hybridization-based methodologies for high throughput mutational analysis using high-density oligonucleotide arrays (DNA chips) have been developed, see Hacia *et al.*, 1996, Detection of heterozygous mutations in BRCA1 using high density oligonucleotide arrays and two-color fluorescence analysis. Nat. Genet. 14:441-447, Hacia *et al.*, New  
20 approaches to BRCA1 mutation detection, Breast Disease 10:45-59 and Ramsey 1998, DNA chips: State-of-Art, Nat Biotechnol. 16:40-44, all incorporated herein by reference for all purposes). Oligonucleotide arrays have been used to screen for sequence

variations in, for example, the *CFTR* gene (U.S. Patent Number 6,027,880, Cronin et al., 1996, Cystic fibrosis mutation detection by hybridization to light-generated DNA probe arrays. Hum. Mut. 7:244-255, both incorporated by reference), the human immunodeficiency virus (HIV-1) reverse transcriptase and protease genes (U.S. Patent Number 5,862,242 and Kozal et al., 1996, Extensive polymorphisms observed in HIV-1  
 5 clade B protease gene using high density oligonucleotide arrays. Nature Med. 1:735-759, both incorporated herein by reference for all purposes), the mitochondrial genome (Chee et al., 1996, Accessing genetic information with high density DNA arrays. Science 274:610-614) and the BRCA1 gene (U.S. Patent Number 6,013,449, incorporated herein  
 10 by reference for all purposes)

## SUMMARY OF THE INVENTION

This invention provides methods and software products for predicting hybridization affinity of a nucleic acid probe against its target. In preferred embodiments, the probe is immobilized on a solid substrate. In most preferred embodiments, the probe  
 15 is part of a nucleic acid probe array, such as synthetic oligonucleotides on a glass substrate, on optical fibers or beads. The methods and software have extensive applications in enhancing mutation detection, selecting probes for probe arrays, and enhancing quality control of probe array manufacturing process.

In one aspect of the invention, computer implemented methods for predicting  
 20 hybridization affinity of a probe against its target is provided. The methods include a step of calculating hybridization intensity (I) using the equation:

$$I = \exp\left[\sum_i P_i S_i\right]$$

wherein  $P_i$  is the value of the  $i$ th parameter and  $S_i$  is a value derived from the sequence of the probe. In some embodiments,  $P_i$  is the free energy of a base in a given position of the sequence relative to a reference base selected from the group consisting of A, C, G and T.

$S_i$  may be a functional of said sequence. The  $P_i$  may be determined empirically, for

example, using least square fit of a training data set.  $P_i$  values may be determined using Cholesky decomposition. In some embodiments,  $S_i$  for a given nucleotide type is replaced with a smooth function of probe base number. The smooth function may be a polynomial function. In some other embodiments, the  $S_i$  for a given nucleotide type is replaced with a smooth function of the difference of a base to the central position.

In some additional embodiments, the equation includes at least one parameter describing probe-probe hybridization and/or at least one parameter describing hairpin formation.

In some other preferred embodiment, the equation includes parameters describing nearest neighbor interactions. In such embodiments,  $P_i$  values may be determined using singular value decomposition.

In another aspect of the invention, computer implemented methods for selecting probes for gene expression monitoring is provided. The methods include a step of predicting hybridization intensities of a plurality of candidate probes and their corresponding control probes; and a step of selecting the candidate probe that has the highest intensity difference over its corresponding control probe. Alternatively, the intensity difference may be directly predicted.

The hybridization intensity ( $I$ ) is determined using the equation:

$$I = \exp\left[\sum_i P_i S_i\right]$$

wherein  $P_i$  is the value of the  $i$ th parameter and  $S_i$  is a value derived from the sequence of the probe. The  $P_i$  is the free energy of a base in a given position of said sequence relative to a reference base selected from the group consisting of A, C, G and T.  $S_i$  is a functional of the sequence of the probe.  $P_i$  is determined empirically, for example, using least

5 square fit of a training data set.  $P_i$  values may be determined using Cholesky decomposition. In some embodiments,  $S_i$  for a given nucleotide type is replaced with a smooth function of probe base number. Exemplary smooth functions include polynomial functions. In some other embodiments,  $S_i$  for a given nucleotide type is replaced with a smooth function of the difference of a base to the central position. In some additional  
10 embodiments, the equation includes at least one parameter describing probe-probe hybridization, at least one parameter describing hairpin formation, and/or parameters describing nearest neighbor interactions. If the equation includes parameters describing nearest neighbor interactions, the  $P_i$  values may be determined using singular value decomposition.

15 In another aspect of the invention, computer software products for predicting hybridization affinity of a probe against its target are provided. The software may be written in any of various suitable computer programming languages such as C, C++, Fortran, Basic, and Java. Software components such as Java Beans, enterprise Java Beans, etc., may be employed as a part of the architecture of the software products of the  
20 invention.

The software products include computer program code for calculating hybridization intensity ( $I$ ) using the equation:



$$I = \exp[ \sum_i P_i S_i ]$$

wherein  $P_i$  is the value of the  $i$ th parameter and  $S_i$  is a value derived from the sequence of the probe and a computer readable media for storing the computer program code. One of skill in the art would appreciate that code for displaying result of the calculation may also

5 be included.  $P_i$  is the free energy of a base in a given position of the sequence relative to a reference base selected from the group consisting of A, C, G and T.  $S_i$  is a functional of the sequence.  $P_i$  values are determined empirically, for example, by least square fit using a training data set, using Cholesky decomposition. In some embodiments,  $S_i$  for a given nucleotide type is replaced with a smooth function of probe base number, such as a  
10 polynomial function. In some other embodiments,  $S_i$  for a given nucleotide type is replaced with a smooth function of the difference of a base to the central position.

In some embodiments, the equation includes at least one parameter describing probe-probe hybridization, at least one parameter describing hairpin formation and/or parameters describing nearest neighbor interactions. If the equation include parameters  
15 describing nearest neighbor interaction,  $P_i$  values are determined using singular value decomposition.

In yet another aspect of the invention, computer software products for selecting probes for gene expression monitoring are provided. The software product include computer program code for predicting hybridization intensities of a plurality of candidate  
20 probes and their corresponding control probes; computer program code for selecting the candidate probe that has the highest intensity difference over its corresponding control

probe; and a computer readable media for storing the codes. The computer code for predicting hybridization intensity (I) including code for calculating using the equation:

$$I = \exp\left[\sum_i P_i S_i\right]$$

wherein  $P_i$  is the value of the  $i$ th parameter and  $S_i$  is a value derived from the sequence of said probe. In some embodiments,  $P_i$  is the free energy of a base in a given position of said sequence relative to a reference base selected from the group consisting of A, C, G and T.  $S_i$  is a functional of the sequence of the probe.  $P_i$  values are determined empirically by, for example, least square fit of a training data set using Cholesky decomposition.  $S_i$  for a given nucleotide type is replaced with a smooth function of probe base number. The smooth function may be a polynomial function. In other embodiments,  $S_i$  for a given nucleotide type is replaced with a smooth function of the difference of a base to the central position. In some embodiments, the equation may include at least one parameter describing probe-probe hybridization, at least one parameter describing hairpin formation and/or parameters describing nearest neighbor interactions. If the equation includes parameters describing nearest neighbor interactions,  $P_i$  values may be determined using singular value decomposition.

## BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and form a part of this specification, illustrate embodiments of the invention and, together with the description, serve to explain the principles of the invention:

Figure 1 illustrates an example of a computer system that may be utilized to execute the software of an embodiment of the invention.

Figure 2 illustrates a system block diagram of the computer system of Fig. 1.

Figure 3a shows measured and fitted intensities for 400 bases (out of a total of 5.6 kb) of the BRCA1 sequence. 97 parameters are used in the fit -- 75 base-type and position parameters (a parameter for each non-T base at each of the 25 probe positions) and one normalization parameter for each of the 22 exons. The separate normalization parameters for each exon allow for the fact that different exons may be present at different concentrations. The root mean square (r.m.s.) deviation of the (natural) logs of  $I_{\text{fit}}$  and  $I_{\text{msd}}$  over the entire sequence is 0.8565. Figure 3b shows the 75 base- and position-specific parameters used to calculate the fitted intensities in Figure 3a.

Figure 4a shows measured and fitted intensities for a portion of the BRCA1 sequence. 37 parameters are used in the fit -- 15 specify the three fourth-degree polynomials describing the base- and position-dependence of the hybridization, and the remaining 22 are normalization parameters for each of the 22 exons. The r.m.s. deviation of the logs of  $I_{\text{fit}}$  and  $I_{\text{msd}}$  over the entire sequence is 0.8712.

Figure 4b shows the base- and position-dependent hybridization free energies derived from the fitting parameters used in Figure 4a. Each curve is a fourth

degree polynomial specified by five of the fitting parameters. These curves are lower information content versions of those shown in Figure 3b.

Figure 5a shows measured and fitted intensities for a portion of the BRCA1 sequence. 39 parameters are used in the fit -- 37 of them are as in Figure 4, and two additional ones describe probe-probe interactions and hairpin formation. The r.m.s. deviation of the logs of  $I_{\text{fit}}$  and  $I_{\text{msd}}$  over the entire sequence is 0.8274.

Figure 5b shows the base- and position-dependent hybridization free energies derived from the fitting parameters used in Figure 5a.

Figure 6a shows measured and fitted intensities for a portion of the BRCA1 sequence. 99 parameters are used in the fit -- 75 specify the 15 fourth-degree polynomials describing the base- and position-dependence of each non-reference nearest-neighbor term, two describe probe-probe interactions and hairpin formation, and 22 are normalization parameters for each of the 22 exons. The r.m.s. deviation of the logs of  $I_{\text{fit}}$  and  $I_{\text{msd}}$  over the entire sequence is 0.7436.

Figures 6b-d show the base- and position-dependent nearest-neighbor hybridization free energies derived from the fitting parameters used in Figure 6a.

Figure 7a shows un-normalized and sequence-based normalized one-color ratios for a portion of the BRCA1 sequence. The sequence-based normalization used the same 37 parameter types used in Figure 5. Ratios above 1.3 are called as mutations. The sequence-based normalization eliminates a false positive just below sequence number 2300 while leaving the signal of the true mutation at position 2798 undiminished.

Figure 7b shows un-normalized and sequence-based normalized one-color ratios for a portion of the BRCA1 sequence. The normalization scheme is the same as in Figure 7a. The sequence-based normalization eliminates several false positives for this wild-type sample. Breaks in the normalized ratio curve occur at exon boundaries.

Figure 8 shows predicted intensity difference is used to select probe pairs with large intensity difference. Intensity differences are predicted using nearest-neighbor parameters derived from a BRCA1 data set. Probe pairs with predicted intensity differences above a cutoff value are selected. Here the cutoff is expressed as a percentile of all predicted intensity differences. A cutoff at the zero percentile corresponds to accepting all probe pairs, regardless of predicted intensity difference. A 50th percentile cutoff corresponds to selecting only those probe pairs with predicted intensity differences in the upper half of all predicted intensity differences. The measured intensity differences are from mRNA hybridized to a gene expression chip. By selecting probe pairs with large predicted intensity difference, one obtains probe pairs with larger actual intensity difference.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Reference will now be made in detail to the preferred embodiments of the invention. While the invention will be described in conjunction with the preferred embodiments, it will be understood that they are not intended to limit the invention to

these embodiments. On the contrary, the invention is intended to cover alternatives, modifications and equivalents, which may be included within the spirit and scope of the invention.

As will be appreciated by one of skill in the art, the present invention may be embodied as a method, data processing system or program products. Accordingly, the present invention may take the form of data analysis systems, methods, analysis software, etc. Software written according to the present invention is to be stored in some form of computer readable medium, such as memory, or CD ROM, or transmitted over a network, and executed by a processor.

Computer software products may be written in any of various suitable programming languages, such as C, C++, Fortran and Java. The computer software product may be an independent application with data input and data display modules. Alternatively, the computer software products may be classes that may be instantiated as distributed objects. The computer software products may also be component software such as Java Beans, Enterprise Java Beans (EJB), Microsoft® COM/DCOM, etc.

Fig. 1 illustrates an example of a computer system that may be used to execute the software of an embodiment of the invention. Fig. 1 shows a computer system 1 that includes a display 3, screen 5, cabinet 7, keyboard 9, and mouse 11. Mouse 11 may have one or more buttons for interacting with a graphic user interface. Cabinet 7 houses a CD-ROM or DVD-ROM drive 13, system memory and a hard drive (*see*, Fig. 2) which may be utilized to store and retrieve software programs incorporating computer code that implements the invention, data for use with the invention and the like. Although a CD

is shown as an exemplary computer readable medium, other computer readable storage media including floppy disk, tape, flash memory, system memory, and hard drive may be utilized. Additionally, a data signal embodied in a carrier wave (*e.g.*, in a network including the internet) may be the computer readable storage medium.

Fig. 2 shows a system block diagram of computer system 1 used to execute the software of an embodiment of the invention. As in Fig. 1, computer system 1 includes monitor 3, keyboard 9, and mouse 11. Computer system 1 further includes subsystems such as a central processor 51, system memory 53, fixed storage 55 (*e.g.*, hard drive), removable storage 57 (*e.g.*, CD-ROM), display adapter 59, sound card 61, speakers 63, and network interface 65. Other computer systems suitable for use with the invention may include additional or fewer subsystems. For example, another computer system may include more than one processor 51 or a cache memory. Computer systems suitable for use with the invention may also be embedded in a measurement instrument.

## **I. Glossary**

“Nucleic acids,” according to the present invention, may include any polymer or oligomer of nucleosides or nucleotides, which include pyrimidine and purine bases, preferably cytosine, thymine, and uracil, and adenine and guanine, respectively. See Albert L. Lehninger, *PRINCIPLES OF BIOCHEMISTRY*, at 793-800 (Worth Pub. 1982). Indeed, the present invention contemplates any deoxyribonucleotide, ribonucleotide or peptide nucleic acid component, and any chemical variants thereof, such as methylated, hydroxymethylated or glucosylated forms of these bases, and the like. The polymers or oligomers may be heterogeneous or homogeneous in composition, and

may be isolated from naturally-occurring sources or may be artificially or synthetically produced. See U.S. patent application Serial No. 08/630,427 which is incorporated herein by reference in its entirety for all purposes. In addition, the nucleic acids may be DNA or RNA, or a mixture thereof, and may exist permanently or transitionally in single-  
5 stranded or double-stranded form, including homoduplex, heteroduplex, and hybrid states. Oligonucleotide and polynucleotide are included in this definition and relate to two or more nucleic acids in a polynucleotide.

“Probe,” as used herein, is defined as a nucleic acid, such as an oligonucleotide, capable of binding to a target nucleic acid of complementary sequence through one or  
10 more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. As used herein, a probe may include natural (i.e. A, G, U, C, or T) or modified bases (7-deazaguanosine, inosine, etc.). In addition, the bases in probes may be joined by a linkage other than a phosphodiester bond, so long as it does not interfere with hybridization. Thus, probes may be peptide nucleic acids in which the  
15 constituent bases are joined by peptide bonds rather than phosphodiester linkages.

“Target nucleic acid” refers to a nucleic acid (often derived from a biological sample), to which the probe is designed to specifically hybridize. It is either the presence or absence of the target nucleic acid that is to be detected, or the amount of the target nucleic acid that is to be quantified. The target nucleic acid has a sequence that is  
20 complementary to the nucleic acid sequence of the corresponding probe directed to the target. The term target nucleic acid may refer to the specific subsequence of a larger nucleic acid to which the probe is directed or to the overall sequence (e.g., gene or



mRNA) whose expression level it is desired to detect. The difference in usage will be apparent from context.

An "array" may comprise a solid support with peptide or nucleic acid probes attached to said support. Arrays typically comprise a plurality of different nucleic acid or peptide probes that are coupled to a surface of a substrate in different, known locations. These arrays, also described as "microarrays" or colloquially "chips" have been generally described in the art, for example, U.S. Pat. Nos. 5,143,854, 5,445,934, 5,744,305, 5,677,195, 6,040,193, 5,424,186 and Fodor et al., Science, 251:767-777 (1991). Each of which is incorporated by reference in its entirety for all purposes. These arrays may generally be produced using mechanical synthesis methods or light directed synthesis methods which incorporate a combination of photolithographic methods and solid phase synthesis methods. Techniques for the synthesis of these arrays using mechanical synthesis methods are described in, e.g., U.S. Pat. Nos. 5,384,261, and 6,040,193, which are incorporated herein by reference in their entirety for all purposes. Although a planar array surface is preferred, the array may be fabricated on a surface of virtually any shape or even a multiplicity of surfaces. Arrays may be peptides or nucleic acids on beads, gels, polymeric surfaces, fibers such as fiber optics, glass or any other appropriate substrate, see U.S. Patent Nos. 5,770,358, 5,789,162, 5,708,153, 6,040,193 and 5,800,992, which are hereby incorporated in their entirety for all purposes. Arrays may be packaged in such a manner as to allow for diagnostics or other manipulation of in an all inclusive device, see for example, US Patent Nos. 5,856,174 and 5,922,591, and 5,945,334, which are incorporated herein in their entirety by reference for all purposes. See also U.S. patent

application Serial No. 09/545,207 which is incorporated herein in its entirety for all purposes for additional information concerning arrays, their manufacture, and their characteristics. It is hereby incorporated by reference in its entirety for all purposes.

## 5 **II. Models and Computational Methods**

This invention is partially based upon a model of the sequence dependence of nucleic acid hybridization. One aspect of the invention provides methods, systems and software products for predicting the hybridization affinity between nucleic acid probes. The methods, systems and software products are particularly useful to enhance loss-of-  
10 signal footprints for detection of heterozygous mutations and to predict probe intensities for the design of gene expression chips. This work should also find wide application in other intensity normalization applications, chip manufacturing quality control, and basic understanding of the thermodynamics of hybridization on a chip.

Hybridization propensities may be described by energetic parameters derived from  
15 the probe sequence, and variations in hybridization and chip manufacturing conditions will result in changes in these parameters which can be detected and corrected.

In one aspect of the invention, models are developed to use energetic parameters to predict or fit nucleic acid hybridization affinity. Nucleic acid hybridization may involve a probe and a target nucleic acid. A probe generally refers to a nucleic acid that is  
20 used to determine qualitatively or quantitatively a target nucleic acid. A target nucleic acid is a nucleic acid of interest. A target molecule hybridizes to a probe with a favorable free energy,  $\Delta G$ , which is a function of the sequence. The hybridization affinity between

the probe and the target molecules may be reflected by certain measurements. For example, if the probe is immobilized on a solid substrate, a target may be labeled with a marker and be contacted with the immobilized substrate to allow hybridization to occur.

The hybridization affinity may be measured by determining the relative level of markers remaining after the substrate is washed. In preferred embodiments, the target molecules are labeled with a fluorescent marker. By determining the fluorescence intensity, the amount of remaining fluorescent marker after the wash, and thus the hybridization affinity, may be determined because the remaining intensity is proportional to the hybridization affinity.

Hybridization affinity may be given by Boltzmann's equation,  $\exp(\Delta G / RT)$ , where T is the absolute temperature and R is the gas constant. Therefore the log of the intensity, I, is linearly related to  $\Delta G$ :

$$\log(I) = c + \Delta G \quad (\text{equation 1})$$

where c is a constant which is proportional to the log of the target concentration, and may be re-scaled to units of RT. (Throughout the specification, "log" refers to the natural log, with base e).

The dependence of  $\Delta G$  on probe sequence can be quite complicated, but relatively simple models for  $\Delta G$  have yielded good results. In general,  $\Delta G$  is a function of the probe sequence of the form:

$$\Delta G(\text{seq}) = \sum_i P_i S_i \quad (\text{equation 2})$$

where the sum runs from 1 to the number of parameters;  $P_i$  is the value of the  $i$ th parameter and  $S_i$  is a corresponding value derived from the probe sequence, as explained below. A function which assigns a scalar value to points in a multi-dimensional space is called a functional of the space. The  $S_i$ 's are functionals of the probe sequence.

- 5 For example, by giving the values of  $S$ , one could use for a 5-mer probe. If one assigns a parameter to each probe sequence position and nucleotide type, for a probe with sequence GACTA, one might try the following values of  $S$ :

Table 1.  $S$  Values

I	position	Base	$S_i$	
1	1	A	0	
2	1	C	0	
3	1	G	1	(1 <sup>st</sup> position is G)
4	1	T	0	
5	2	A	1	(2 <sup>nd</sup> position is A)
6	2	C	0	
7	2	G	0	
8	2	T	0	
9	3	A	0	
10	3	C	1	(3 <sup>rd</sup> position is C)
11	3	G	0	
12	3	T	0	

13	4	A	0	
14	4	C	0	
15	4	G	0	
16	4	T	1	(4 <sup>th</sup> position is a T)
17	5	A	1	(5 <sup>th</sup> position is an A)
18	5	C	0	
19	5	G	0	
20	5	T	0	
21			1	(one more value – this value is set to one for all sequences, and the corresponding parameter will be an over-all normalization)

The model and data permits the estimation of relative free energies. In mathematical terms, the above choice for S over-specifies the sequences and results in singular equations. Each probe position has one of four nucleotide types, so there are only three degrees of freedom per probe position. There are many possible ways around this problem. In some embodiments, the problem is solved by choosing one nucleotide type as a reference and using fewer parameters. If base T is chosen as a reference, S values are as follows:

Table 2. S Values When T is a Reference

I	position	Base	S <sub>i</sub>	
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1	1	A	0	
2	1	C	0	
3	1	G	1	(1 <sup>st</sup> position is G)
4	2	A	1	(2 <sup>nd</sup> position is A)
5	2	C	0	
6	2	G	0	
7	3	A	0	
8	3	C	1	(3 <sup>rd</sup> position is C)
9	3	G	0	
10	4	A	0	
11	4	C	0	
12	4	G	0	(4 <sup>th</sup> position is T - which now has no corresponding parameter)
13	5	A	1	(5 <sup>th</sup> position is A)
14	5	C	0	
15	5	G	0	
16			1	(for over-all normalization)

In this representation, the Pi's will be the free energy of a base in a given position relative to a T in the same position.

With a functional form chosen for  $\Delta G$  (seq) i.e., with a set of functionals  $S_i$  chosen), one can determine the values of the parameters which give the best fit to the observed intensities in a set of data from a chip. The fitted intensity  $I_{fit}$  is of the form

$$I_{fit} = \exp [\sum_i P_i S_i] \quad (\text{equation 3})$$

Where the sum is over all the parameters  $P_i$  and  $S_i$  are the corresponding functionals of the probe sequence. The  $P_i$  are chosen to minimize the quantity

$$\sum_{\text{probes}} \{ \log (I_{fit}) - \log (I_{msd}) \}^2 = \sum_{\text{probes}} \{ \sum_i P_i S_i - \log (I_{msd}) \}^2 \quad (\text{equation 4})$$

where  $I_{msd}$  is the measured intensity, the  $P_i$  are parameters to be determined and the  $S_i$  are obtained from each probe sequence. This is a least-squares fit to the logs of the measured intensities. By choosing  $I_{fit}$  as above, a linear least-square approach may be used to estimate the parameters.

The least-squares fit to determine the  $P_i$  is computationally tractable for the tens of thousands of data points from a typical chip by direct solution of the normal equations using Cholesky decomposition (*see*, for example, Gene H. Golub and Charles F. van Loan: Matrix Computations, 2nd edn., The John Hopkins University Press, 1989, incorporated herein for all purposes). While some numerical methods texts warn against direct solution of the normal equations, this inventor has found them to be well-behaved

even in single precision if the parameters are properly scaled. In preferred embodiments, double precision was used and no numerical problems were observed.

In some embodiments, the hybridization intensities, which is a measurement of hybridization affinity, are predicted using equation:  $I = \exp[\sum_i P_i S_i]$ .

5 The  $P_i$  values are obtained using, for example, a training data set. Once the  $P_i$  values are obtained, the hybridization intensities of a probe is calculated using the equation 1. One of skill in the art would appreciate that the  $P_i$  values may be dependent upon hybridization conditions, such as hybridization and subsequent wash temperature, ionic strength, etc. Therefore, a given set of  $P_i$  values may only be applicable to a given hybridization condition.

## **II. Refinements to the model**

One of skill in the art would appreciate that there are many possible variations on the hybridization model outlined above. The first is the use of polynomial fits to reduce the number of parameters required to describe the data. The second is the addition of parameters describing hairpin formation to the target molecule. The third refinement is the use of "nearest neighbor" parameters to improve the description of hybridization.

### *A. Reduction of number of parameters*

Depending on the particular application and the size of the data, it may be desirable to reduce the number of parameters in the fit. One way of reducing the parameter is to replace the set of parameters for a given nucleotide type with a smooth function of probe base number. To return to the 5-mer example and the probe with sequence GACTA, one can choose to fit to second degree polynomials. The energetic



parameters for each nucleotide type are taken to be quadratic functions of the base number. The values of  $S$  would be:

Table 3.  $S$  Values

I	Base	degree	$S_i$	
1	A	0	2	(there are two A's)
2	A	1	$2 + 5$	(an A in position 2 and one in position 5)
3	A	2	$4 + 25$	(again, A at 2 and another at 5. This is $2^2 + 5^2$ )
4	C	0	1	(one C)
5	C	1	3	(C is in position 3)
6	C	2	9	( $3^2$ )
7	G	0	1	(one G)
8	G	1	1	(in position 1)
9	G	2	1	( $1^2$ )
10			1	(for normalization)

- 5 This representation reduces the number of parameters for 5-mer probes from 16 to 10.

For numerical reasons, one preferred implementation differs slightly from the one given above. The fit is made not to probe position but to the difference from the central position, and Legendre polynomials are used rather than a simple power series.

- 10 For 25-mer probes, in some preferred embodiments, 4<sup>th</sup> degree Legendre polynomials were used, resulting in 16 total parameters, as opposed to 76 without the polynomial fit.

## B. *Probe-probe hybridization and hairpin information*

When data are fit with the above prescription, there are prominent regions where the fitted and measured intensities disagree. Many of these regions are due to probe-probe hybridization or hairpin formation in either the target or the probes. In one aspect of the invention, a probe-probe score and a hairpin score are used to describe the effects of these phenomena on hybridization intensity. The two scores differ in that sequences must have a loop of at least four bases to form a hairpin, whereas there is no such requirement for the bi-molecular probe-probe interaction.

## C. *Nearest neighbor parameters*

Hybridization free energies depend not only on the identity of the bases involved, but also on their order. In particular, there are significant stacking effects between successive bases. These effects are called "nearest neighbor" interactions, and they can be made quantitative by assigning different hybridization free energies to different dinucleotide types. Thus DNA - RNA hybridization is usually described with 16 distinct free energies for AA, AC, AG, AT, CA, ..., TG, TT. DNA-DNA hybridization (in solution) requires only 8 parameters due to the symmetry between the two strands.

Applying a nearest neighbor description to fit hybridization data requires a change in the mathematics. If the above models are extended to include nearest-neighbor effects, and assign parameters to AA, AC, AG, AT, CA, ..., TC, TG (and leave TT as a reference) for positions 1-2, and then the same for all positions up to 24-25, the resulting normal equations are singular. This is because the parameters describe a space larger than that of

true sequences. For example, it is not possible to simultaneously have AA in positions 1-2 and GG in positions 2-3, yet the parameters can describe such a situation.

One solution is to solve the normal equations with singular value decomposition rather than Cholesky decomposition. In effect, the computer constructs a basis set which excludes the null space which corresponds to impossible sequences, and finds the optimal solution in the remaining lower-dimensional space.

In one aspect of the invention, computer software products are provided to perform the hybridization intensity prediction. The software products contains code that performs the prediction using the equation:  $I = \exp[\sum_i P_i S_i]$ . The code may be stored in a suitable media and executed in a suitable digital computer. In some embodiments, the equation may be modified to account for probe-probe interaction, hairpin formation, nearest neighbor effect. The equation may also be modified to reduce the number of parameters as described above.

The software may include code that inputs  $S_i$ , which are parameters related to the sequence of a probe. The assignment of  $S$ -values are illustrated in tables 1-3. The software may also include code for inputting  $P_i$  values, which are determined empirically as disclosed above. In addition, the software may include code for displaying resulting intensity value for a probe. In one exemplary software product, the software inputs the sequence of a probe, code are executed to assign  $S$  values based upon the sequence,  $P_i$  values are inputted from a relational database or a flat file. Intensity values are calculated and outputted to a display or for use of components of the software. For example, the

intensity values may be used for probe selection (described below) in another module of the software product.

### **III. Application to mutation detection**

5 In one aspect of the invention, the model of hybridization developed above, methods and computer software for predicting nucleic acid hybridization are modified to correct for variations in hybridization or manufacturing conditions between two nucleic acid probe arrays. In some embodiments, the methods and computer software are used to improve detection of mutations, particularly heterozygous mutations. In some preferred  
10 embodiments, the methods and software may be used to improve the analysis of a loss-of-signal assay sensitive to all possible mutations (substitutions, insertions and deletions) of a given wild-type sequence. However, the methods and software may also be applied to detecting gain of signal if the mutant type is tiled for (for a more detailed description of tiling and mutation detection, *see, e.g.,* . Methods for designing, selecting and making  
15 tiling probe sets are described in, for example, WO 95/11995, published on May 4, 1995, incorporated herein by reference for all purposes. Hacia *et al.* (1996, Detection of heterozygous mutations in BRCA1 using high density oligonucleotide arrays and two-color fluorescence analysis. Nat. Genet. 14:441-447) describe a loss-of-signal assay which utilizes on- and off-chip references and two distinct fluorescent labels. In this  
20 "two-color" assay, the unknown target sample is labeled with one type of fluorescent label (e.g. red) while a wild-type reference is labeled with a second fluorescent label (e.g., green). On a second chip, wild-type target is labeled with both red and green and

hybridized to the chip. For each perfect-match probe that is designed to be complementary to a target, a ratio of intensities is calculated:

$$\text{ratio} = [I(1, \text{wt, green}) I(2, \text{wt, red})] / [I(1, \text{unk, red}) I(2, \text{wt, green})] \quad (\text{equation 4})$$

where  $I(1, \text{unk, red})$  is the intensity of the unknown target on chip 1,  $I(1, \text{wt, green})$  is the intensity of the wild-type reference on the same chip,  $I(2, \text{wt, red})$  is the intensity of the red-labeled wild-type sample on the reference chip, and  $I(2, \text{wt, green})$  is the intensity of the green-labeled wild-type sample on the reference chip. The ratio is constructed so as to cancel out effects of chip-to-chip variation and effects due to differences between the two types of fluorescent labels. Hacia et al. then took a five-position moving window average of this ratio to signal the presence of a mutation. Values of the window-averaged ratio above a threshold were taken to indicate mutations.

The sequence-based normalization scheme involves fitting the ratio, either one-color or two-color, with the energetic parameters described above. Differences in the hybridization conditions are largely canceled out when the sequence-normalized ratio

$$\text{normalized ratio} = \text{ratio} * \exp [ -[\sum_i P_i S_i] ] \quad (\text{equation 5})$$

is formed, where  $P_i$  is the  $i$ th parameter of the fit to the ratio and  $S_i$  is the corresponding value or functional of the probe sequence. Another way of expressing this is to say that the normalized ratio is the measured ratio divided by the fit to the ratio (i.e. a residual

ratio). This normalization results in a large improvement of the performance of the one-color assay, and a more modest improvement in the performance of the two-color assay. Results of the one-color assay using sequence-based normalization yielded 6 out of 7 true positives in 7 samples, each consisting of 35 exons and 5.5 kb of sequence of the MSH2 and MLH1 genes. These results suggest that the method may be very useful in its present form as a preliminary screen, greatly limiting the amount of gel-based sequencing required. With a new chip design incorporating multiple copies of perfect match probes, further improvements in performance are possible.

The two-color assay performs very well without sequence-based normalization. The error rates seem low enough that it will require a fairly large amount of data (probably several hundred samples) to specify them, and also to specify what improvement sequence-based normalization will yield. Nevertheless, based on signal-to-noise ratios, sequence-based normalization seems likely to decrease the error rates of the two-color assay as well.

#### **IV. Application to probe selection for gene expression**

The models developed here can yield predictions of probe intensities which are useful for selecting probes for gene expression.

In one implementation of the gene expression monitoring arrays (see, U.S. Patent Nos. 5,800,992 and 6,040,138, incorporated herein by reference for all purposes). Each gene is interrogated by a group of probe pairs. Each probe pair consists of a probe (perfect

match) that is designed to be complementary to its target and a mismatch probe that is designed to contain a mismatch against its target. The intensity difference of each probe pair (perfect match minus mismatch) is predicted using the models described above.

Figure 8 shows the effectiveness of this strategy. The data is from yeast mRNA

5 hybridized to a gene expression chip. The intensity difference is predicted based on a nearest-neighbor fit to BRCA1 data. The plot shows the effects of using predicted intensity difference as a means of selecting probes for the design of gene expression chips. Probe pairs with large intensity difference are desired. A zero percentile cutoff means all probe pairs are included, while a 50<sup>th</sup> percentile cutoff means that only those  
10 probe pairs with predicted intensities in the upper half of the predictions are included. A factor of two increase in actual intensity difference is easily obtained by this method.

## **V. Application to chip manufacturing quality control**

In general the parameters and hybridization intensities will also reflect variations in the chip manufacturing process. A variation in the coupling efficiency of a synthesis  
15 step should have a multiplicative effect on probe intensity, like variations in hybridization free energy. For example, a synthesis step with 0.9 coupling efficiency followed by one with 0.85 efficiency results in 0.9 times 0.85 of the probes successfully completing both synthesis steps. As is the case with energetic terms, only relative efficiencies can be detected by the methods presented here, not absolute efficiencies.

20 For chips manufactured with the shift-mask process, energetic parameters corresponding to probe sequence number and base type also correspond to specific cycles in the manufacturing process. The parameters in figure 1b are an example of this case. If

there were a synthesis step which was substantially less efficient than the others, one would expect a dip at the parameter corresponding to that step in figure 1b. For chips not made with shift-masks, parameters corresponding to specific cycles could also be constructed. Cycles with low yield should be detectable by relatively low values in the corresponding parameters derived from fitting the measured intensities.

### Examples

Some embodiments of the methods of the invention were investigated using data from experiments examining mutation in the BRCA1 gene.

Germline mutations in BRCA1 are present in 50-60% of kindreds with breast and ovarian cancer, and may account for approximately 2-5% of all breast cancer cases in the general population (Hall et al., *Science* 250, 1684-1689 (1990), Narod et al., *Lancet* 338, 82-83 (1991), Easton et al. *Am. J. Hum. Genet.* 52, 678-701 (1993), Rowell et al., *Am. J. Hum. Genet.* 55, 861-865 (1994)). Heterozygous carriers are markedly predisposed to early onset breast and ovarian cancer, and are also at moderately increased risk of developing colon and prostate cancer (Ford et al., *Lancet* 343, 692-695 (1994)). The protein coding region of BRCA1 contains 5,592-bp in 22 coding exons spread over 100-kb of genomic DNA (Miki et al., *Science* 266, 66-71 (1994)). Over 111 unique BRCA1 mutations distributed throughout the gene have been described in the literature (Shattuck-Eidens et al., *Am. Med. Assoc.* 273, 535-541 (1995) and (Breast Cancer Information Core Database located on the *World Wide Web* at <http://www.nchgr.nih.gov/Intramural-research/Lab-transfer/Bic/>)). Most of these are frameshift, nonsense, or splice mutations resulting in a disruption of the normal reading frame. Except for the Ashkenazi Jewish



population, where two mutations account for the majority of BRCA1 alterations

(Struwing et al., *Am J. Hum. Genet.* 57, 1-7 (1995); Struwing et al., *Nature Genet.* 11, 198-200 (1995); Tonin et al., *Am. J. Hum. Genet.* 57, 189 (1995); Friedman et al., *Am. J. Hum. Genet.* 57, 1284-1297 (1995); FitzGerald et al., *N. Engl. J. Med.* 334, 143-149

(1996); Offit et al., *Lancet* 347, 1643-1645 (1996)), allelic heterogeneity confounds the ability to identify BRCA1 mutation carriers by methods (such as allele-specific oligonucleotide [ASO] hybridization) which detect only a finite set of previously described mutations.

#### A. Methods

##### (a) PCR from Genomic DNA and RNA Target Preparation

PCR reactions were performed on genomic samples using the EXPAND™ Long Range PCR Kit (Boehringer Mannheim) with primers 11FT3 5'-ATTAACCCTCACTAAAGGGAATTAAATGAAAGAGTATGAGC-3' and 11RT7 5'-TAATACGACTCACTATAGGGAGTGCTCCCAAAGCATAAA-3' containing T3 and T7 RNA polymerase promoter sequences respectively. In vitro transcription reactions from these exon 11 amplicon templates were performed in 10 µl reaction volumes using T3 RNA polymerase transcription buffer (Promega), 0.7 MM of ATP, CTP, GTP, and UTP, 10 MM DTT, 0.7 MM fluorescein-12-UTP or 0.15 MM biotin-16-UTP (Boehringer Mannheim) for reference and test samples respectively, and 10U T3 or T7 RNA polymerase as indicated.

##### (b) Target Preparation and Analysis

A reference template was generated from PCR amplification of exon 11 from a *BRCA1* cDNA clone. Reference and test sample transcription products were diluted to a final concentration of 100 nM in a 25 µl solution of 30 mM MgCl<sub>2</sub>. The reaction was incubated at 94°C for 70 minutes to fragment targets (Lipshutz, et al., *BioTechniques* 19, 442-447 (1995); Kozal, et al., *Nature Med.* 2, 753-759 (1996)). Cofragmented targets were diluted 1/100 into a 300 µl volume of hybridization buffer (3 M TMAC-Cl (tetramethylammonium chloride), 1x TE pH 7.4, 0.001% Triton X-100, 1 nM 5'-fluorescein-labelled control oligonucleotide 5'-CGGTAGCATCTTGAC-3'). This control oligonucleotide is designed to hybridize to specific surface probes to aid in image alignment. Target was hybridized with the chip in a 250 µl volume for 4 hours at 35°C. The chip surface was washed with 10 ml of wash buffer (6X SSPE, 0.001% Triton X-100) and stained with phycoerythrin-streptavidin conjugate (Molecular Probes) (2 µg/ml in wash buffer) for 5 minutes at room temperature. The chip was washed with 10 ml of wash buffer and scanned as described (Lipshutz et al., *BioTechniques* 19, 442-447 (1995); Kozal et al., *Nature Med.* 2, 753-759 (1996)). Hybridization signals were detected by a photomultiplier tube using 515-545 nm bandpass and 560 nm longpass emission filters for fluorescein reference (green) and biotin test (red) samples respectively (Cronin et al., *Hum. Mut.* 7, 244-255 (1996); Chee et al., *Science* 274, 610-614 (1996)).

#### (d) Dideoxysequencing analysis

Four pairs of PCR primers (P1M13+ 5'-GTTTCCCAGTCACACGGAATTAAATGAAAG AGTATGAGC-3' and P1M13- 5'-AGGAAACAGCTATGACCATGTGAGGGGACGCTCT TG-3', P2M13+ 5'-

GTTTCCCAGTCACACGTTGGGAAAACCTATCGGAA-3' and P2M135'-

AGGAAACAGCTATGACCATCTTTGGGGTCTTCAGCA-3', P3M13+ 5'-

GTTTCCCAG TCACACGTGTTCAAATACCAGTGAACCTTA-3' and P3M13- 5'-

AGGAAACAGCTATG ACCATGGAGCCCACT7CATTAGTAC-3', P4M13+ 5'-

5 GTTTCCCAGTCACACGCCAAGT ACAGTGAGCACAATTA-3' and P4M13- 5'-

AGGAAACAGCTATGACCATGTGCTCCC AAAAGCATAAA-3') were designed to generate four partially overlapping amplicons which cover the entire sequence of exon 11 and contain M13 forward and reverse sequences at the 5'-end of either strand Depending on region to be analyzed one of the four amplicons was generated from the appropriate genomic sample using the EXPAND™ Long Range PCR kit (Boehringer Mannheim) with the recommended protocol. Dye primer dideoxysequencing reactions were performed using AmpliTaq DNA Polymerase FS kit (Perkin Elmer) with the suggested protocol and either M13 forward or M13 reverse DYEnamic™ energy transfer dye primers (Amersham Life Science).

#### (d) Data Analysis

Photomultiplier output signal was converted into proportional spatially addressed pixel values using GeneChip® Software (Affymetrix, Inc., Santa Clara, CA) to create a digitized fluorescence image. The relative contributions of the reference and test targets to each probe signal were extracted from each set of experimental green reference and red test images and imported into a Microsoft Excel 7.0a worksheet.

## **B. Results**

### (a) Prediction of Hybridization Affinity

Figure 3a shows a subset of experimental data from a BRCA1 chip and the fitted (predicted) intensities. Measured and fitted intensities (a measurement of hybridization affinity) for 400 bases (out of a total of 5.6 kb) of the BRCA1 sequence are shown. The fitted parameters are shown in Figure 3b. 97 parameters are used in the fit -- 75 base-type

5 and position parameters (a parameter for each non-T base at each of the 25 probe positions) and one normalization parameter for each of the 22 exons. The separate normalization parameters for each exon allow for the fact that different exons may be present at different concentrations. The root mean square (r.m.s.) deviation of the (natural) logs of  $I_{fit}$  and  $I_{msd}$  over the entire sequence is 0.8565, which indicates a good fit.

10 The parameters in Figure 3b have a simple interpretation. For example a C in the 10<sup>th</sup> position has a value of about 0.46. This means that the log of  $I_{fit}$  for a probe with a C in the 10<sup>th</sup> position will be 0.46 greater than the log of  $I_{fit}$  for a probe with a T in the 10<sup>th</sup> position and the same bases in all 24 other positions. In this data, where the target is RNA, one can see by examining Figure 3b that C's in the probe generally have the most

15 favorable hybridization free energy, followed by G's, A's, and then T's.

The shapes of the curves in Figure 3b are in rough agreement with the shapes of footprints due to point mutations (or "smile plots"). As is seen with footprints, the curves peak near the middle of the probe sequence, indicating that positions near the middle have the largest effects on hybridization.

## 20 (b) Reduction of Parameters

Figure 4b shows the base- and position-dependent hybridization free energies derived from the fitting parameters used in Figure 4a. Each curve is a fourth degree

polynomial specified by five of the fitting parameters. 37 parameters are used in the fit -- 15 specify the three fourth-degree polynomials describing the base-and position-dependence of the hybridization, and the remaining 22 are normalization parameters for each of the 22 exons. These curves are lower information content versions of those shown in Figure 3b. The r.m.s. deviation of the logs of  $I_{\text{fit}}$  and  $I_{\text{msd}}$  over the entire sequence is 0.8712. In spite of the large reduction in the number of parameters, the quality of the fit is degraded very little.

#### (c) Probe-Probe Hybridization and Hairpin Formation

Figures 5a and 5b are the same as Figures 4a and 4b, except that parameters corresponding to the probe-probe and hairpin scores were also included in the fit. 39 parameters are used in the fit -- 37 of them are as in Figure 4, and two additional ones describe probe-probe interactions and hairpin formation. The r.m.s. deviation of the logs of  $I_{\text{fit}}$  and  $I_{\text{msd}}$  over the entire sequence is 0.8274. One region where the fit improves is around sequence number 1980. Examination of the sequence around position 1980 shows that both probe-probe interactions and hairpins are likely there.

#### (d) Nearest Neighbor Parameters

Figures 6a-d show the results of using singular value decomposition approach using a 4<sup>th</sup> degree polynomial fit to the nearest-neighbor free energies. 99 parameters are used in the fit -- 75 specify the 15 fourth-degree polynomials describing the base- and position-dependence of each non-reference nearest-neighbor term, two describe probe-probe interactions and hairpin formation, and 22 are normalization parameters for each of the 22 exons. The r.m.s. deviation of the logs of  $I_{\text{fit}}$  and  $I_{\text{msd}}$  over the entire sequence is

0.7436. Figures 6b-d show the base- and position-dependent nearest-neighbor hybridization free energies derived from the fitting parameters used in Figure 6a.

#### (e) Application in Mutation Detection.

Figure 7a shows the effect of sequence-based normalization on the one-color ratio for a portion of the sequence of BRCA1. The sequence-based normalization reduces the signal from potential false positives, while the signal from the true mutation at position 2798 is not diminished. Figure 7b shows the elimination of false positives by sequence-based normalization of a wild-type BRCA1 sample.

#### (d) Probe Selection for Gene Expression

Figure 8 shows the effectiveness of probe selection based upon predictions of probe intensities. The data is from yeast mRNA hybridized to a gene expression chip. The intensity difference of each probe pair (perfect match minus mismatch) is predicted based on a nearest-neighbor fit to BRCA1 data. The plot shows the effects of using predicted intensity difference as a means of selecting probes for the design of gene expression chips. Probe pairs with large intensity difference are desired. A zero percentile cutoff means all probe pairs are included, while a 50<sup>th</sup> percentile cutoff means that only those probe pairs with predicted intensities in the upper half of the predictions are included. A factor of two increase in actual intensity difference is easily obtained by this method.

### **Conclusion**

The present inventions provide methods and computer software products for predicting nucleic acid hybridization affinity, detecting mutation, selecting probes,

and improving probe array manufacturing quality control. It is to be understood that the above description is intended to be illustrative and not restrictive. Many variations of the invention will be apparent to those of skill in the art upon reviewing the above description. By way of example, the invention has been described primarily with  
5 reference to the use of a high density oligonucleotide array, but it will be readily recognized by those of skill in the art that the methods may be used to predict the hybridization affinity of other immobilized probes, such as probes that are immobilized in or on optical fibers or other supports. The basic methods and computer software of the invention may also be used to predict solution based hybridization. The scope of the  
10 invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled.

What is claimed is:

1. A computer implemented method for selecting probes for gene expression monitoring comprising:

- 5 a) predicting hybridization intensities of a plurality of candidate probes and their corresponding control probes; and
- b) selecting the candidate probe that has the highest intensity difference over its corresponding control probe.

10 2. The method of Claim 1 wherein said hybridization intensity (I) is determined using the equation:

$$I = \exp \left[ \sum_i P_i S_i \right]$$

wherein said  $P_i$  is the value of the  $i$ th parameter and  $S_i$  is a value derived from the sequence of said probe.

15 3. The method of Claim 2 wherein said  $P_i$  is the free energy of a base in a given position of said sequence relative to a reference base selected from the group consisting of A, C, G and T.

20 4. The method of Claim 3 wherein said  $S_i$  is a functional of said sequence.

5. The method of Claim 3 wherein said  $P_i$  is determined empirically.



6. The method of Claim 5 wherein said  $P_i$  is determined by least square fit using a training data set.

7. The method of Claim 5 wherein  $P_i$  is determined using Cholesky decomposition.

8. The method of Claim 7 wherein said  $S_i$  for a given nucleotide type is replaced with a smooth function of probe base number.

9. The method of Claim 8 wherein said smooth function is a polynomial function.

10. The method of Claim 7 wherein said  $S_i$  for a given nucleotide type is replaced with a smooth function of the difference of a base to the central position.

11. The method of Claim 8 wherein said smooth function is a polynomial function.

12. The method of Claim 7 wherein said equation includes at least one parameter describing probe-probe hybridization.

13. The method of Claim 7 wherein said equation includes at least one parameter describing hairpin formation.

14. The method of Claim 7 wherein said equation includes parameters describing nearest neighbor interactions.

15. The method of Claim 7 wherein said  $P_i$  is determined using singular value decomposition.

16. A computer implemented method for predicting hybridization affinity of a probe against its target comprising calculating hybridization intensity (I) using the equation:

$$I = \exp\left[\sum_i P_i S_i\right]$$

wherein said  $P_i$  is the value of the  $i$ th parameter and  $S_i$  is a value derived from the sequence of said probe.

17. The method of Claim 16 wherein said  $P_i$  is the free energy of a base in a given position of said sequence relative to a reference base selected from the group consisting of A, C, G and T.

18. The method of Claim 17 wherein said  $S_i$  is a functional of said sequence.

19. The method of Claim 18 wherein said  $P_i$  is determined empirically.

20. The method of Claim 19 wherein said  $P_i$  is determined by least square fit using a training data set.

21. The method of Claim 20 wherein  $P_i$  is determined using Cholesky decomposition.

22. The method of Claim 21 wherein said  $S_i$  for a given nucleotide type is replaced

5 with a smooth function of probe base number.

23. The method of Claim 22 whereon said smooth function is a polynomial function.

24. The method of Claim 21 wherein said  $S_i$  for a given nucleotide type is replaced

10 with a smooth function of the difference of a base to the central position.

25. The method of Claim 24 wherein said smooth function is a polynomial function.

26. The method of Claim 21 wherein said equation includes at least one parameter

15 describing probe-probe hybridization.

27. The method of Claim 21 wherein said equation includes at least one parameter  
describing hairpin formation.

20 28. The method of Claim 20 wherein said equation includes parameters describing  
nearest neighbor interactions.

29. The method of Claim 28 wherein said  $P_i$  is determined using singular value decomposition.

30. The method of Claim 16 wherein said probe is immobilized on a solid substrate.

5

31. A computer software product for predicting hybridization affinity of a probe against its target comprising:

computer program code for calculating hybridization intensity (I) using the equation:

$$I = \exp\left[\sum_i P_i S_i\right]$$

wherein said  $P_i$  is the value of the  $i$ th parameter and  $S_i$  is a value derived from the sequence of said probe; and

a computer readable media for storing said computer program code.

32. The computer software product of Claim 31 wherein said  $P_i$  is the free energy of a base in a given position of said sequence relative to a reference base selected from the group consisting of A, C, G and T.

33. The computer software product of Claim 32 wherein said  $S_i$  is a functional of said sequence.

34. The computer software product of Claim 33 wherein said  $P_i$  is determined empirically.

35. The computer software product of Claim 34 wherein said  $P_i$  is determined by least  
5 square fit using a training data set.

36. The computer software product of Claim 35 wherein  $P_i$  is determined using Cholesky decomposition.

37. The computer software product of Claim 36 wherein said  $S_i$  for a given nucleotide  
10 type is replaced with a smooth function of probe base number.

38. The computer software product of Claim 37 wherein said smooth function is a  
polynomial function.

39. The computer software product of Claim 36 wherein said  $S_i$  for a given nucleotide  
15 type is replaced with a smooth function of the difference of a base to the central position.

40. The computer software product of Claim 39 wherein said smooth function is a  
20 polynomial function.

41. The computer software product of Claim 36 wherein said equation includes at least one parameter describing probe-probe hybridization.

42. The computer software product of Claim 36 wherein said equation includes at least one parameter describing hairpin formation.

43. The computer software product of Claim 35 wherein said equation includes parameters describing nearest neighbor interactions.

44. The computer software product of Claim 18 wherein said  $P_i$  is determined using singular value decomposition.

45. A computer software product for selecting probes for gene expression monitoring comprising:

computer program code for predicting hybridization intensities of a plurality of candidate probes and their corresponding control probes;

computer program code for selecting the candidate probe that has the highest intensity difference over its corresponding control probe; and

a computer readable media for storing said codes.

46. The computer software product of Claim 45 wherein said computer code for predicting hybridization intensity ( $I$ ) comprising code for calculating using the equation:

$$I = \exp[ \sum_i P_i S_i ]$$

wherein said  $P_i$  is the value of the  $i$ th parameter and  $S_i$  is a value derived from the sequence of said probe.

5 47. The computer software product of Claim 46 wherein said  $P_i$  is the free energy of a base in a given position of said sequence relative to a reference base selected from the group consisting of A, C, G and T.

48. The computer software product of Claim 47 wherein said  $S_i$  is a functional of said  
10 sequence.

49. The computer software product of Claim 47 wherein said  $P_i$  is determined empirically.

15 50. The computer software product of Claim 49 wherein said  $P_i$  is determined by least square fit using a training data set.

51. The computer software product of Claim 50 wherein  $P_i$  is determined using Cholesky decomposition.

20

52. The computer software product of Claim 51 wherein said  $S_i$  for a given nucleotide type is replaced with a smooth function of probe base number.

53. The computer software product of Claim 52 whereon said smooth function is a polynomial function.

5 54. The computer software product of Claim 52 wherein said  $S_i$  for a given nucleotide type is replaced with a smooth function of the difference of a base to the central position.

55. The computer software product of Claim 52 wherein said smooth function is a polynomial function.

10 56. The computer software product of Claim 51 wherein said equation includes at least one parameter describing probe-probe hybridization.

15 57. The computer software product of Claim 51 wherein said equation includes at least one parameter describing hairpin formation.

58. The computer software product of Claim 51 wherein said equation includes parameters describing nearest neighbor interactions.

20 59. The computer software product of Claim 58 wherein said  $P_i$  is determined using singular value decomposition.



## ABSTRACT

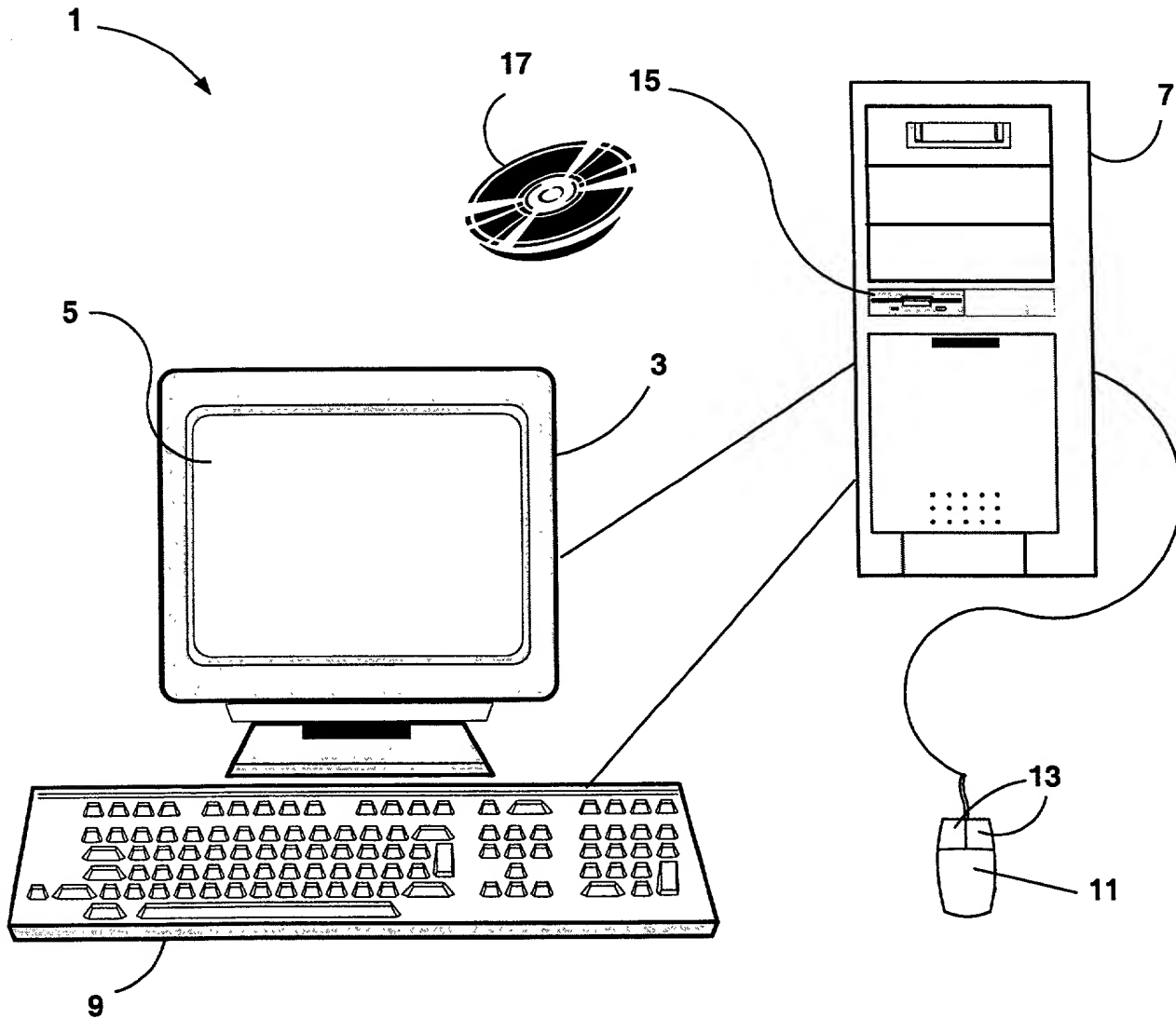
Methods and computer software products are provided for predicting nucleic acid hybridization affinity. In one embodiment, hybridization intensity (I) is determined using

5 the equation:

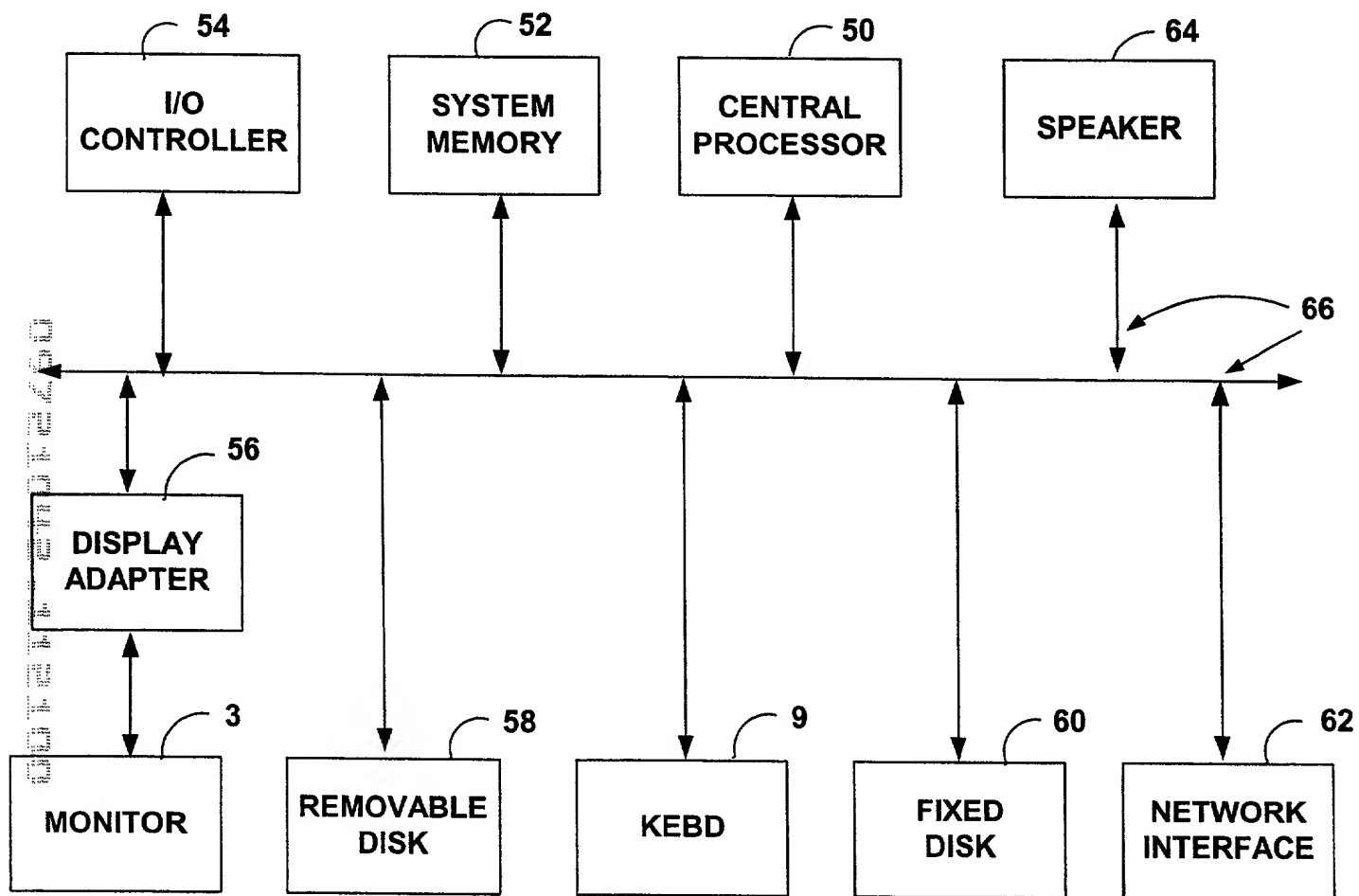
$$I = \exp\left[\sum_i P_i S_i\right]$$

where  $P_i$  is the value of the  $i$ th parameter and  $S_i$  is a value derived from the sequence of the probe. The methods and software products of the invention may be used for enhancing mutation detection, probe selection and probe array manufacturing quality

10 control.



**Figure 1**



**Figure 2**

**Figure 3a**

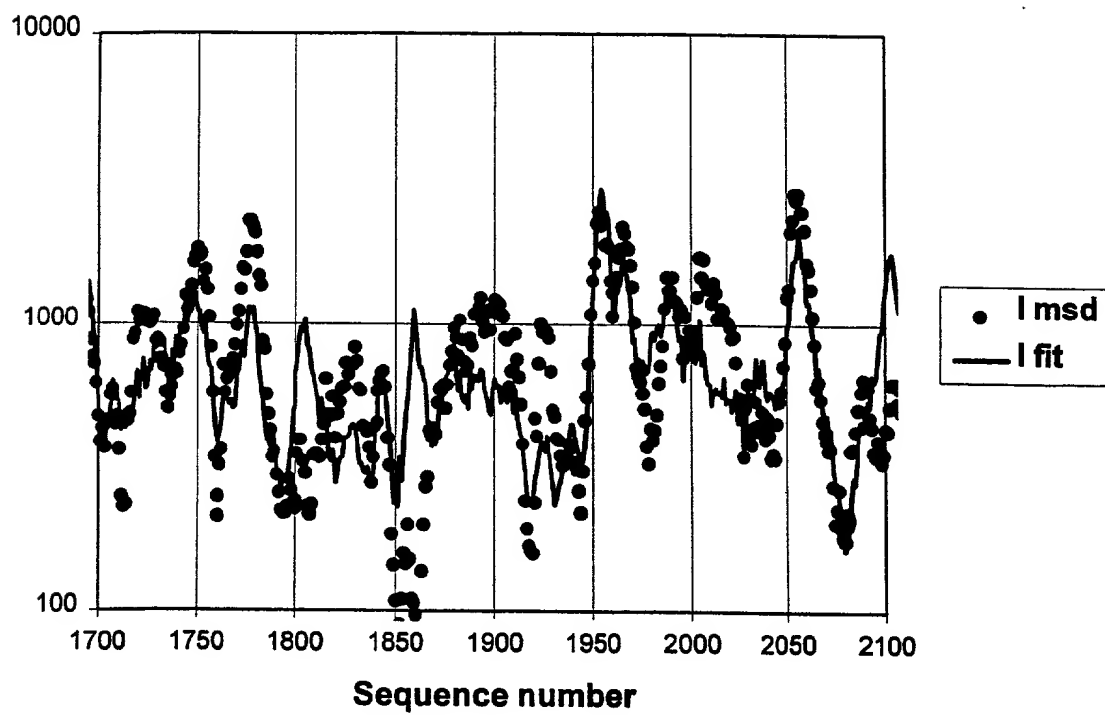


Figure 3b

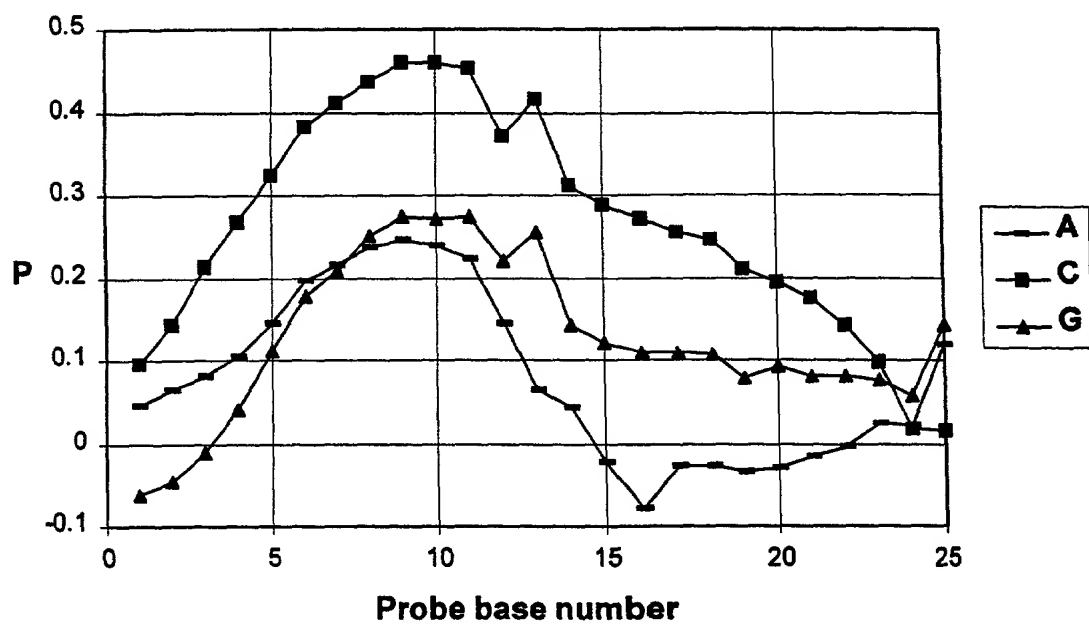
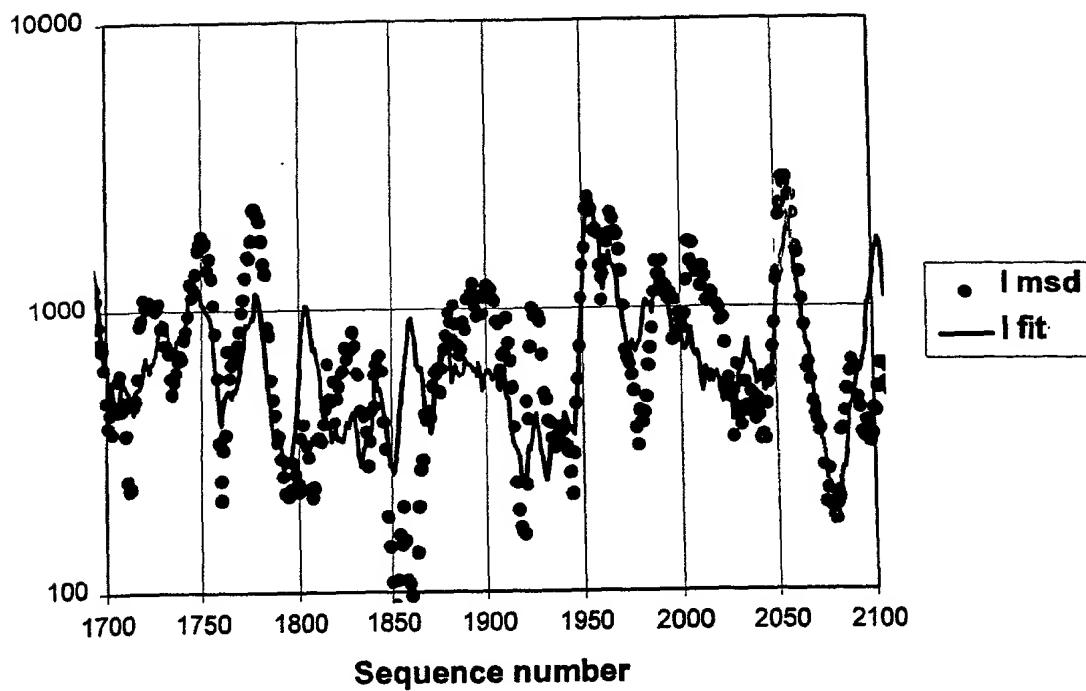


Figure 4a



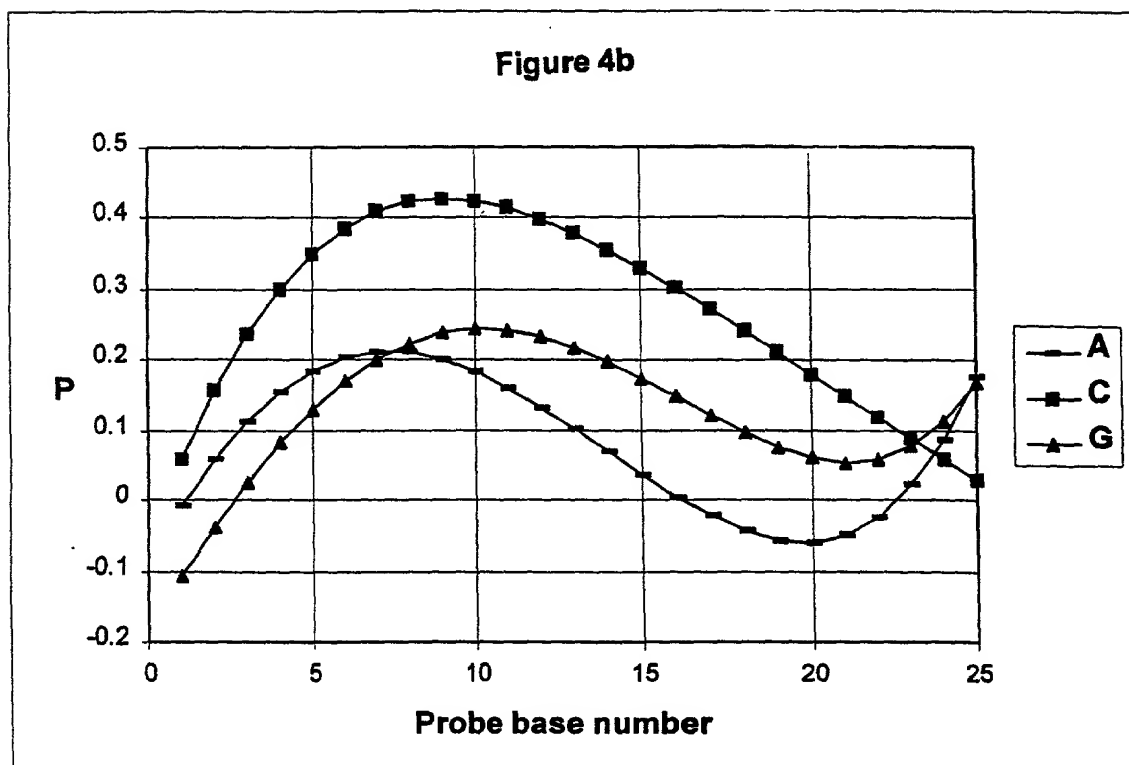
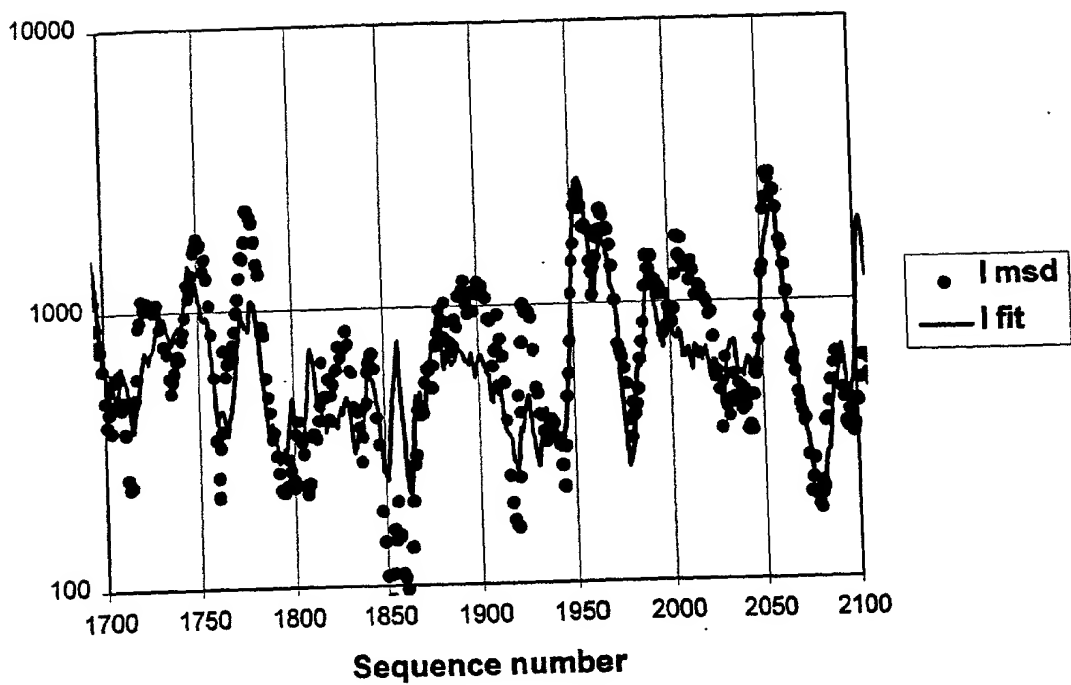


Figure 5a





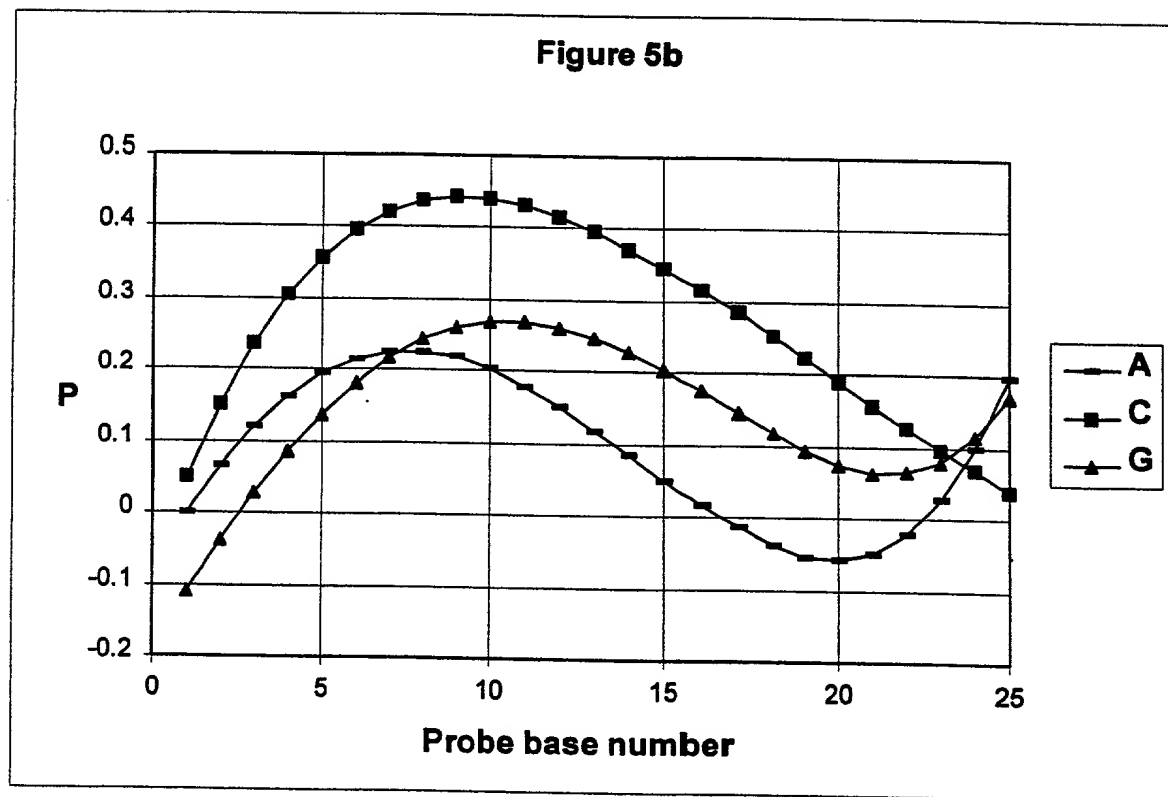


Figure 6a

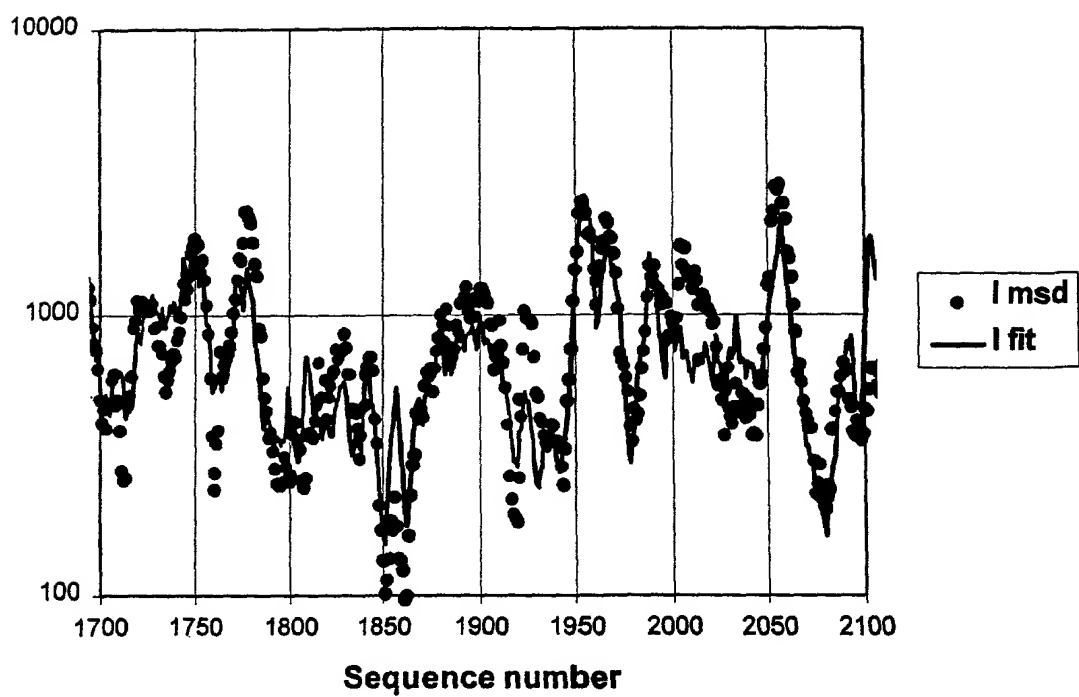


Figure 6b

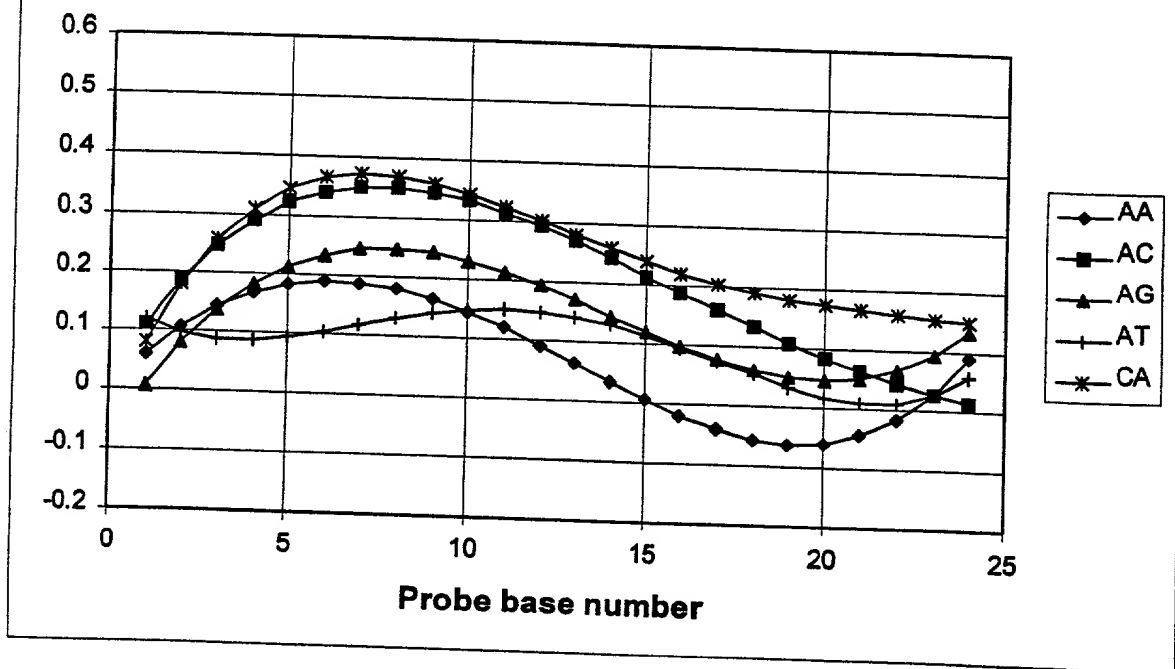


Figure 6c

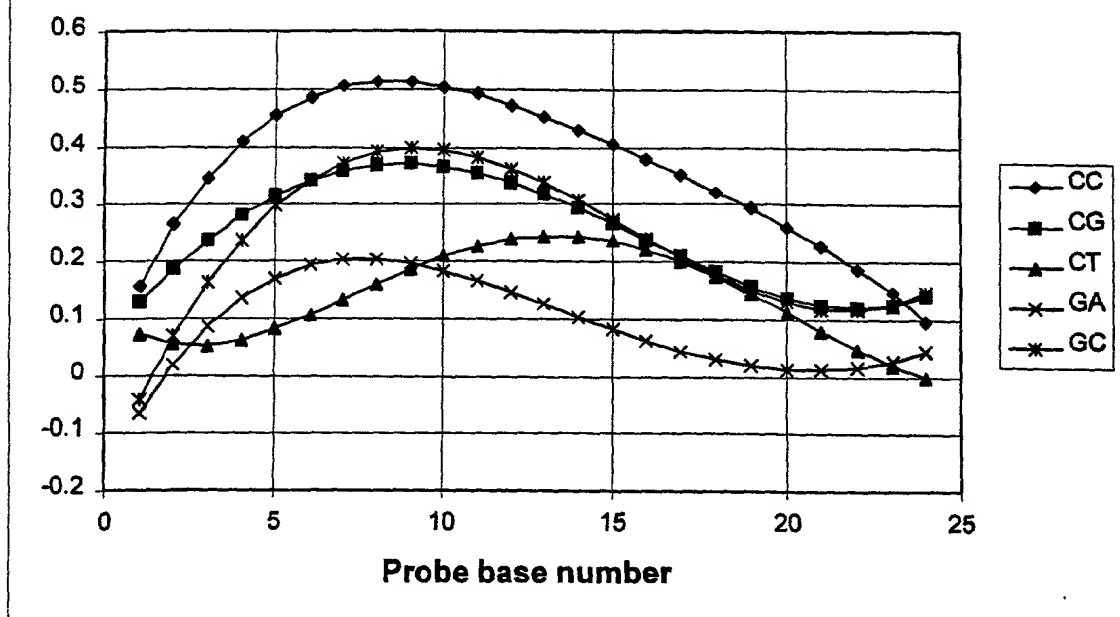


Figure 6d

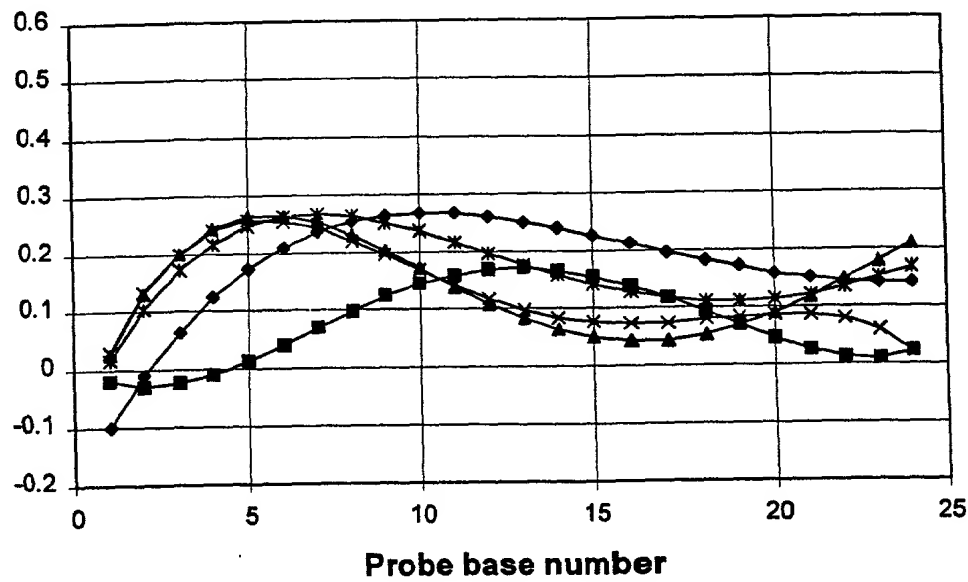


Figure 7a

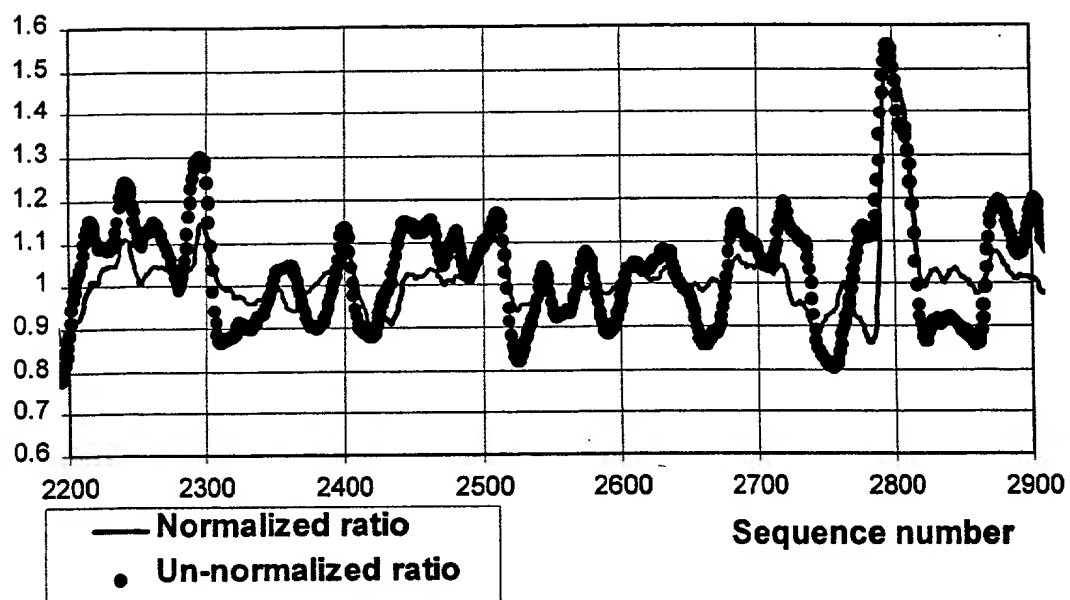
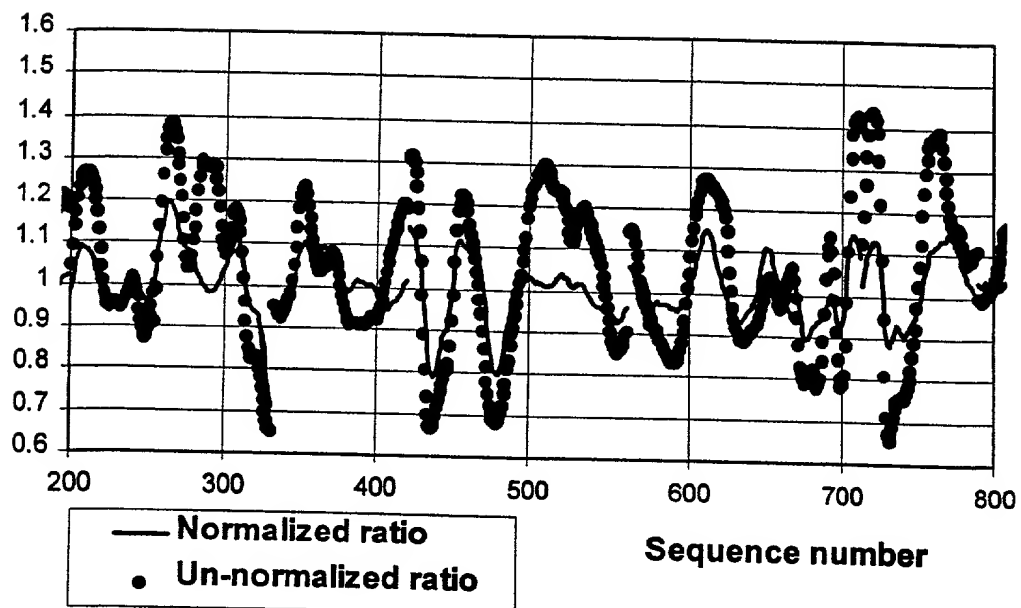
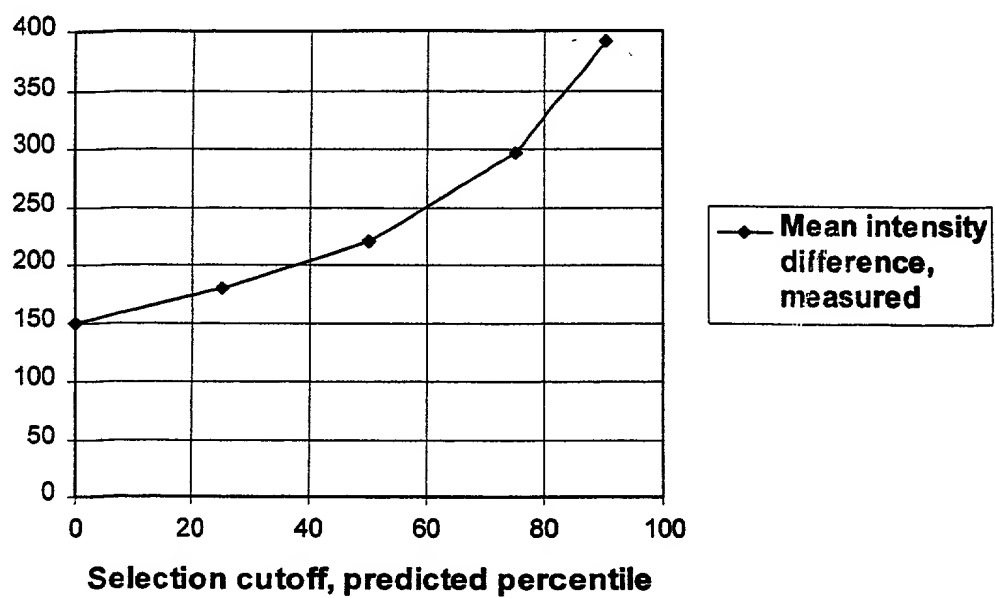


Figure 7b



**Figure 8**





Docket No.

3367

# Declaration and Power of Attorney For Patent Application

## English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

### METHODS AND COMPUTER SOFTWARE PRODUCTS FOR PREDICTING NUCLEIC ACID HYBRIDIZATION AFFINITY

the specification of which

(check one)

☒ is attached hereto.

☐ was filed on \_\_\_\_\_ as United States Application No. or PCT International  
Application Number \_\_\_\_\_  
and was amended on \_\_\_\_\_

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Status)  
(patented, pending, abandoned)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Status)  
(patented, pending, abandoned)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Status)  
(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

**POWER OF ATTORNEY:** As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

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Sole or first inventor's signature

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Second inventor's signature

Date

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